Marijana Pećarević*

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APPLICATION OF MOLECULAR GENETIC METHODS FOR DETECTION OF HARMFUL ORGANISMS IN SHIP'S BALLAST WATER

Primjena metoda molekularne genetike za uočavanje štetnih organizama u brodskoj balastnoj vodi

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Summary

Non-native species are spread around the globe by various means, including aquaculture and ship vectors, hull fouling and ballast water. The introduction of non-indigenous species may cause a serious harm to ecosystems, economy and human health. The ballast water is a primary source for the introduction of harmful species and pathogens, therefore, the detection of such organisms could prevent unwanted impacts.

The use of molecular methods may provide focused detection with high throughput of samples, in a time and cost effective manner. A major advancement in the study of ballast water microorganisms will be to expedite sample analysis using a sensitive oligonucleotide hybridization technique, the technology of DNA microarrays. Microarray technology would be powerful in detecting microbial pollution within any coastal or inland waters used for transport, recreation or drinking.

Sažetak

Alohtone vrste prenose se diljem svijeta različitim sredstvima, najčešće u obraštaju ili balastnim tankovima brodova te akvakulturnim djelatnostima. Unos stranih vrsta može imati štetne učinke na ekosustav, gospodarstvo ili ljudsko zdravlje. Opasne vrste i patogeni prenose se većinom u vodenom balastu pa bi njihovo pravodobno otkrivanje moglo spriječiti štetne posljedice.

Korištenje molekularnim metodama moglo bi omogućiti istovremenu obradu više uzoraka i brzu detekciju ciljanih vrsta bez velikih troškova. Najveći napredak u istraživanju mikroorganizama u vodenom balastu ostvaren je uporabom osjetljive tehnologije genskih čipova (DNA microarrays). Ta tehnologija bit će izuzetno korisna i za otkrivanje patogena u balastnim vodama, jednako kao i u vodama za rekreaciju i piće.

* **Marijana Pećarević, dipl. ing., znanstveni novak, Sveučilište u Dubrovniku**

Introduction

Uvod

Biological invasions are increasingly recognized as a primary threat to global biodiversity (Bax et al. 2001). Uncontroled invasions of non-indigenous species can result in unwanted ecological, economic, animal and and human health impacts.

Invaders arrive by various means, including aquaculture and ship vectors, hull fouling and ballast water (Gollasch, 2002). However, ballast water transfer by oceangoing vessels has been identified as a leading invasion pathway (Carlton and Geller, 1993). Ballast water is pumped and gravitated into vessel at one port and discharged at another, transporting planktonic, nektonic and benthic organisms between biogeographic regions.

Microorganisms are especially abundant in ballast water; therefore, the potential for global transfer of microorganisms in ballast tanks is enormous and may represent a serious risk for dispersal of pathogens (Ruiz et al. 2000). Challenge and important task for scientists in port states around the globe is the detection of such organisms in a time and cost effective manner to help

minimize discharge of contaminated water. Conventional methods of detection involve specialized techniques or expertise that are not available in all laboratories. Therefore, robust sampling of microorganisms currently involves collecting samples, then distributing them to diverse laboratories, each capable of determining one or several species' presence. A major advancement in the study of ballast water microorganisms, and of aquatic microbial ecology in general, will be to expedite sample analysis using a sensitive oligonucleotide hybridization technique, specifically, the elegant technology of DNA microarrays (Aridgides at al. 2004).

Polymerase Chain Reaction (PCR)

Lančana reakcija polimerazom

The polymerase chain reaction has revolutionized molecular biology over the last ten years. PCR is quick, easy method for generating unlimited copies of any fragment of DNA.

PCR requires a template molecule (DNA or RNA) and two primer molecules to get the copying process started. Primers must be duplicates of nucleotide sequences on either side of the piece of DNA of interest, which means that the exact order of the primers' nucleotides must already be known (Henson and French, 1993; Erlich, 1989, 1999).

There are three basic steps in PCR. First, the target genetic material must be denatured; the strands of its helix must be unwound and separated-by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus newly assembled complementary strand. These PCR steps are repeated for around 30 or 40 cycles, and this is done on an automated PCR thermal cycler or PCR machine (Erlich, 1989, 1999).

PCR accelerated and simplified procedures previously performed much more laboriously by traditional molecular cloning and it quickly found use in experimental molecular biology. A new technology now promises to advance biotechnology even further, and that technology is microarray analysis.

DNA Microarrays

DNA mikročipovi

DNA microarrays, also called DNA chips (or gene-chips), have a major impact on molecular biology. Microarray technology aims to monitor thousands of genes simultaneously on a single chip. The fundamental basis of DNA microarrays is the process of hybridization, where two strands of nucleic acid, target and probe, hybridize if they are complementary to each other. The probe sequences are immobilized on the surface, at a separation of a few micrometers so that is possible to place many different probes on a very small surface (e.g. 1 cm^2). The sample is usually labeled with a fluorescent dye that can be detected by a light scanner that scans the surface of the chip (Fodor et al. 1993).

DNA microarrays are glass or nylon surfaces with multiple spots, each containing a single-stranded oligonucleotide. These oligonucleotides act as hybridization probes for labeled nucleic-acid targets. When microarrays are challenged with samples containing fluorescently labeled target sequences, and when hybridization conditions are appropriate, hybridization occurs between probe and target. The DNA chip is then scored for locations in which fluorescent tags appear. Such a tag confirms the presence of a DNA or RNA target sequence in the sample, indicative of a specific strain, species, or other taxon (Fodor et al. 1993; Ekins and Chu, 1999).

DNA microarrays are fabricated by high-speed robotics, generally on glass but also on nylon substrates, for which probes with known identity are used to determine complementary binding, thus allowing parallel gene expression and gene discovery studies. An experiment with a single DNA chip can provide researchers information on thousands of genes simultaneously (Fodor et al. 1993; Ekins and Chu, 1999).

Microarray technology would be powerful in detecting microbial pollution within any coastal or inland waters used for transport, recreation or drinking.

Recent Experiments and Possible Use of Molecular-Biology Techniques to Simu-ltaneously Detected Organisms in Ballast Water

Noviji pokusi i mogućnosti korištenja metoda molekularne biologije za brzo određivanje organizama u vodenom balastu

First step in designing experiments for multiple detections is to obtain sequence information to design appropriate primers and probes. After testing of probe fidelity (i.e., cross-reactivity with closely related and unrelated organisms), methodical testing of potential interferences, such as competition for probe sites during hybridization and complicating issues of secondary and tertiary structures, must be done. Particular attention must be paid to discriminating between presence of DNA and RNA and viability of target organisms. It is important to optimize chips so that the maximum number of sites may be scored.

Aridgides et al. (2004) conducted proof-of-concept experiments to optimize simultaneous detection of multiple microorganisms using polymerase chain reaction (PCR) and Southern hybridization. Three model organisms were chosen, all potentially found in ballast water: a primate calcivirus (*Pan-1*), the bacterium *Vibrio cholerae* (a non-toxic strain), and the photosynthetic algae *Aureococcus anophagefferens*. Project databases

were created by using information from GenBank (National Center for Biotechnology Information). The PCR primers and Southern hybridization probes were designed to meet two requirements: (1) the primer and probe sets for all organisms had similar melting temperatures and (2) the primer and probe sequences were not homologous to other organisms. Multiplex PCR was performed simultaneously with calcivirus *Pan-1* (cDNA), *Vibrio cholerae* (DNA) and *Aureococcus anophagefferens* (DNA). Multiplex reverse transcription-PCR was successful for *Pan-1* and *A. anophagefferens*. Southern hybridization was performed on the PCR products using digoxiginen labeled probes complimentary for sequences within the PCR products. The positive Southern hybridization results for *Pan-1* as well as *A. anophagefferens* confirmed that both pathogens could be detected simultaneously using traditional methods.

Rublee et al. (2005) used several methods in order to identify and study the distribution of *Pfiesteria piscicida* and *P. scumwayae*, toxic dinoflagellates frequently present in ballast tanks. Light microscope and histochemical methods have been used to identify *Pfiesteria* cells in samples, but these methods are not species specific and therefore cannot be applied in samples where dinoflagellates with similar morphologies may be present. Molecular tools have been especially advantageous since even under "bloom" conditions, *Pfiesteria* cells may constitute only 10% of the total phytoplankton numbers. After developing PCR primers in collaborative studies based on sequence determination from bona fide *P. piscicida* cultures, analytical time was reduced to 1-2 days, dependent on the DNA extraction method used prior to PCR amplification. This was soon followed by development of real-time PCR protocols which reduced time of sample analysis even more. When combined with commercial DNA extraction protocols the time for analysis of water samples could be only 2-3 hours. Application of the molecular methods has demonstrated a worldwide distribution of *Pfiesteria piscicida* and *P. scumwayae*. Genetic variability among geographic locations generally appears low in rDNA genes and detection of the organisms in ballast water is consistent with rapid dispersal of high gene flow among populations.

Discussion

Rasprava

The use of traditional molecular-biology techniques to simultaneously detect ballast-water microorganisms from different phylogenetic groups is a first step toward building complex "smart sensors" with a capacity for high throughput and cost-efficiency analyses. The critical aspect in the design of microarrays and other smart sensors is availability of sequence information in order to test homology with other organisms; otherwise, appropriate primers and probes cannot be designed. The lack of specificity of the primers and probes ultimately led to inability to detect organisms alone or in combination with the other organisms. However, optimized primers and probes enable simultaneous detection of organisms in liquid matrices of ballast water (Aridgides et al. 2004).

An advantage of PCR-based approaches for detection is that the amplified fragments can also be used in sequence analysis, either by generating clones from amplified fragments or by direct sequencing. Knowing the sequence allows insight into additional questions regarding variability among isolates across geographic or temporal ranges, phylogenetic position, distribution and source location of the target organism (Rublee et al. 2005).

DNA microarrays, combined with PCR, are already being used to detect bacterial pathogens in environmental water samples (Call et al. 2003). Future researches using molecular-based technology will be able to identify presence and abundance of organisms in ballast water. The use of molecular methods may provide focused detection with high throughput of samples, in a time and cost effective manner. The implied requirement for multiple samples from tank and ships for effective monitoring and risk assessment programs emphasizes the need for rapid and accurate methods of analysis. Future studies and developing of molecular-biology based methods will allow us to predict the patterns and consequences of biological invasions and better manage the risks of introducing non-indigenous species.

References

Izvori

Aridgides LJ, Doblin MA, Berke T, Dobbs FC, Matson DO, Drake LA (2004) Multiplex PCR allows simultaneous detection of pathogens in ships' ballast water. Marine Pollution Bulletin 48: 1096-1101

Bax N, Carlton JT, Mathews-Amos A, Haedrich RL, Hogwarth FG, Purcell JE, Rieser A, Gray A (2001) The control of biological invasions in the world's oceans. Conservation Biology 15: 1234–1246

Call DR, Borucki MK, Loge FJ (2003) Detection of bacterial pathogens in environmental samples using DNA microarrays. Journal of Microbiological Methods 53: 235–243

Carlton JT, Geller JB (1993) Ecological roulette: the global transport of nonindigenous marine organisms. Science 261: 78-82

Ekins R, Chu FW (1999) Microarrays: their origins and applications. Trends in Biotechnology 17: 217-218

Erlich HA (1999) Principles and applications of the polymerase chain reaction. Review Immunogenetic 1(2):127-34

Erlich HA (1989) PCR Technology: Principles and Applications for DNA Amplification. Stockton Press, New York

Fodor SPA, Rava RP, Huang XC, Pease AC, Holmes CP, Adams CP (1993) Multiplexed biochemical assays with biological chips. Nature 364: 555–556

Gollasch S (2002) The importance of ship hull fouling as a vector of species introductions into the North sea. Biofouling 18: 105-121

Henson JM, French R (1993) The polymerase chain reaction and plant diseases diagnosis. Annual Review of Phytopathology 31: 81-109

Rublee PA, Remington DL, Schaefer EF, Marshall MM (2005) Detection of the Dinozoans *Pfiesteria piscicida* and *P. scumwayae*: A Review of Detection Methods and Geographic Distribution. Journal of Eukaryotic Microbiology 52(2): 83-89

Ruiz GM, Rawlings TK, Dobbs FC, Drake LA, Mullady T, Huq A, Colwell RR (2000) Global spread of microorganisms by ships – Ballast water discharged from vessels harbours a coctail of potential pathogens. Nature 408: 49-50

URL: http://users.ugent.be/~avierstr/principles/pcrani.html

URL:http://www.bio.davidson.edu/courses/genomics/chip/chip.html

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